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Cystic Fibrosis and the gastro-intestinal tract

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CHAPTER 7

GENERAL DISCUSSION AND SUMMARY

M. Wouthuyzen-Bakker

CYSTIC FIBROSIS AND THE GASTRO-INTESTINAL TRACT

Gastro-intestinal disease is, after pulmonary manifestation, the second most severe expression of cystic fibrosis (CF) and includes pancreatic insufficiency, intestinal abnormalities and hepatobiliary disease. The aim of this thesis was to address various aspects of the gastro-intestinal tract in CF disease by evaluating current treatment and investigating potential future treatment options. We evaluated the effect of a high dietary energy intake on the nutritional status in CF patients with preserved pulmonary function (**Chapter 2**), we reviewed potential factors that may contribute to persistent fat malabsorption and we evaluated several treatments options to improve fat absorption and/or nutritional status in CF conditions (**Chapter 3 & 4**). In **Chapter 5**, we investigated in mice *in vivo* the choleretic properties of ursodeoxycholic acid (which is the current medical treatment for CF-related liver disease) under CFTR deficient conditions. Finally, we tested the hypothesis that the severe gallbladder phenotype in CF pigs, compared with CF mice, is due to reduced activity of calcium activated chloride channels (in particular TMEM16A) (**Chapter 6**) which may strengthen the basis for exploiting pharmacological activation of these channels in CF patients.

NUTRITIONAL STATUS IN CYSTIC FIBROSIS

Most patients with CF have a suboptimal nutritional status due to increased energy expenditure (mostly as the result of recurrent pulmonary infections and/or chronic inflammation), insufficient energy intake, and fat malabsorption.[1] Since the nutritional status and the pulmonary function of CF patients are inversely related,[2-4] the degree of malnutrition (often defined as z-scores for weight or BMI < -2) is closely related to morbidity and mortality.[5-6] CF children with malnutrition often show a rapid decline in pulmonary function, which consequently results in a further worsening of the nutritional status and pulmonary function.[2-3] An optimal nutritional status should therefore be maintained to avoid the vicious cycle of pulmonary deterioration. The definition of an optimal nutritional status that should be achieved in CF conditions remains debatable. Z-scores for weight, length and BMI for age are all shown to be associated with pulmonary function.[3] From the CF foundation patient registry data, it has been shown that a z-score for BMI above zero is necessary to maintain a nearly normal pulmonary function; e.g. FEV1 values above 80% of predicted.[4] Therefore, the American CF foundation recommends to maintain z-scores for BMI above zero in CF patients between two and twenty years of age.[7] Based on these observations, a nutritional classification scoring system has been developed to screen and classify patients according to their 'nutritional risk.' Patients with a z-score for BMI below zero are classified into a higher risk category and are more eligible for nutritional intervention compared to patients with higher z-scores.[7] In contrast with these reports, we showed, in our population of pre-school CF patients with preserved pulmonary function (FEV1>80%), that 50% of this cohort of patients have a z-score for BMI below zero (**Chapter 2**). Thus, our data indicate that a z-score for BMI above zero is not essential to maintain a nearly normal pulmonary function in pre-school CF patients. In agreement with our data, McDonald

et al. suggested that z-scores for BMI below zero are justified in young CF patients and a more stringent cut-off value for BMI levels are necessary for older children (above 15 years) in order to maintain a good pulmonary function. [7] In addition to the z-scores for BMI, Konstan et al. showed that only z-scores for weight for age below minus two at three years of age are associated with a deteriorated pulmonary function at the age of six.[3] In our cohort of pre-school CF patients with preserved pulmonary function, only five percent of patients exhibit z-scores for weight below minus two (**Chapter 2**). Thus, only a small fraction of young CF patients with preserved pulmonary function would require nutritional intervention to optimize their nutritional status. For CF patients that do exhibit impairments in pulmonary function, a more stringent approach to improve nutritional status would be necessary. In contrast to CF patients with mild pulmonary disease who would be expected to catch-up in growth rapidly after a pulmonary exacerbation, patients with impaired pulmonary function often have difficulties to regain weight after a pulmonary exacerbation.[1]

Improving a suboptimal nutritional status in CF patients remains an important and difficult challenge in CF care. Since the nutritional status of CF patients is determined by multiple factors affecting the balance between energy requirements and energy expenditure, several targets for optimizing the nutritional status should be addressed. The nutritional status is likely to improve by aggressive pulmonary treatment, by high dietary energy intakes and by improving the fat absorption.[2-3] In regard to the gastro-intestinal tract, pancreatic enzyme replacement therapy in case of pancreas insufficiency and a high energy diet are classic corner stones in the nutritional care for CF patients.

Dietary energy intake

In the late eighties, Corey et al. studied the survival of CF patients in two different CF clinics and observed a better survival in the clinic where a non-restricted, instead of restricted, fat diet was applied (together with a more aggressive strategy towards indications for pulmonary infections). [8] This study has led to a major shift in the nutritional care for CF patients. Instead of a restricted fat diet, a high energy diet with ~40% fat became generally advised to CF patients.[2-3] Despite the reported beneficial effects on nutritional status, the dietary approach also resulted in negative psychological consequences for CF patients and their families to apply to the dietary advice.[9-11] Several studies indicated that most CF patients are not able to meet the nutritional recommendations.[12] We therefore questioned whether a less stringent dietary advice might be justified in relatively healthy CF patients. In **Chapter 2**, we retrospectively analyzed the relation between energy intake and several parameters for nutritional status and growth in a relatively homogeneous subgroup of 81 pediatric CF patients between six and nine years of age with a preserved pulmonary function (FEV1% > 80%). Cross-sectional analysis revealed a lack of correlation between total energy intake or the intake of fat, expressed as the amount of kilocalories intake per kilogram body weight, and several parameters for nutritional status and growth.

Most studies reported a relation between energy intake and nutritional status in malnourished patients (e.g. z-scores for nutritional status < -2), in patients with impaired pulmonary function (FEV1% < 80%), and in patients with initial energy intakes below 100% of the recommended daily allowance for healthy peers.[13-26] The results of these studies suggest that only CF patients who are in a more advanced stage of disease and with dietary intakes that are clearly below the general advice, even for the healthy reference population, quantitatively benefit from high energy intakes. We additionally analyzed the relation between energy intake and weight gain in a longitudinal setting and categorized patients according to their initial nutritional status and energy intake. We found that only a small percentage (9%) of patients who increased the energy intake in the year after the initial assessment, showed a corresponding increase in the z-score for body weight. In contrast, 26% of patients showed, despite a decrease in energy intake, an increase in the z-score for body weight. These results were irrespective of the initial nutritional status or energy intake. We cannot exclude that the increase in energy intake was not high enough to measure a quantitative beneficial effect on weight. Nevertheless, our data do accentuate that energy intake and growth are not always linearly related in CF patients due to the multiple factors which are involved in the maintenance of adequate growth, especially in patients without overt malnutrition. We do realize that our results are based on a retrospective longitudinal analysis, with the risk of bias and with the limitation of three-day dietary diaries as a measurement of energy intake. Our data certainly justifies the need for prospective clinical trials in this subgroup of patients. There are no prospective studies that evaluated the quantitative effect of energy intake on the nutritional status in a subset of patients which are in a relatively healthy state of CF lung disease. These studies should preferably be carried out with the inclusion of energy expenditure measurements and other parameters for nutritional status that were not assessed in our study, like fat mass and fat free mass. These studies are important to conclude whether the actual beneficial effects on nutritional status or growth outweigh the possible adverse psychological consequences in the strive to achieve high dietary energy intakes.

Fat absorption

The physiological process of fat absorption involves various sequential steps. Each step requires a specific environmental condition - e.g. sufficient amount of digestive enzymes and bile salts, adequate bicarbonate secretion and a healthy intestinal mucosa - to ensure adequate fat digestion and absorption (**Chapter 3**).[27] The major contributor to fat malabsorption in CF patients is the insufficient production of the digestive enzyme pancreatic lipase due to pancreatic insufficiency. Treatment with pancreatic enzyme supplements greatly improves fat absorption in CF patients, but in approximately 50% of CF patients fat malabsorption persists.[28] The persistent fat malabsorption may be attributed to insufficient use of pancreatic enzyme supplements, but can also be attributed to several other CF-related gastro-intestinal tract abnormalities. Insight into the contribution of these abnormalities to fat malabsorption in CF conditions is

necessary to ultimately implement new treatment strategies and improve the nutritional status of CF patients.

In **Chapter 3**, we reviewed several factors that have been proposed in the literature to contribute to CF-related fat malabsorption and the corresponding intervention studies in CF patients and CF mice. We found that a reduced intestinal pH due to decreased pancreatic and duodenal bicarbonate secretion may contribute to persistent fat malabsorption in individual CF patients and that therefore, fat absorption may improve during treatment with acidic suppressive drugs.[29] Treatment of intestinal mucus accumulation, chronic inflammation and bacterial overgrowth of the small intestine by broad-spectrum antibiotics and oral laxatives improved the body weight of CFTR knockout mice.[30-33] Whether a similar beneficial effect of these treatments on body weight can be expected in CF patients remains to be determined, especially considering the fact that CFTR knockout mice have a more severe intestinal phenotype compared to CF patients. However, small intestinal bacterial overgrowth has been reported in approximately 40% of CF patients, although the definitive diagnosis has remained a challenge[34-35] Nevertheless, patients with signs compatible wth small bacterial overgrowth (like diarrhoea, bowel gas and weight loss) could benefit from an empirical treatment with broad-spectrum antibiotics. Alterations in essential fatty acids and intestinal transit time are described in CF patients and may theoretically contribute to fat malabsorption[36-37], but the clinical relevance of these factors still needs to be determined. EFA supplements could be considered to improve nutritional status in CF patients, as they have shown to improve body weight in individual cases. Whether the improvement is due to the EFA itself or to a quantitatively higher energy intake in patients using supplements is not clear from the reviewed studies.[38-39] Results from patients as well as animal studies clearly indicated that increased fecal loss of bile salts and alterations in bile salt composition do not contribute to fat malabsorption in CF.[40-43]

Antibiotic treatment has shown to improve the body weight of CFTR knockout mice.[30-33] To determine whether the improved body weight could be attributed to increased absorption of fat, we treated CFTR knockout and CFTR homozygous delta F508 mice with oral antibiotics (metronidazole and ciprofloxacin). After treatment, we determined fat absorption by a 72-hour fecal fat test and determined fatty acid kinetics by intra-gastrically administration of stable isotope-labelled fats (**Chapter 4**). Because the CF mouse models used are pancreas sufficient, these mice are especially suited to study persistent fat malabsorption (mimicking the condition of CF patients with persistent fat malabsorption despite optimal treatment with pancreatic enzyme supplements). We confirmed that the CFTR knockout mice, with a severe intestinal phenotype and small intestinal bacterial overgrowth, improved in body weight after antibiotic treatment, but we also demonstrated that the mechanism is not based on enhancing the fat absorption. The CFTR homozygous delta F508 mice, with a milder intestinal phenotype without small intestinal bacterial overgrowth, showed no improvement in body weight and no quantitatively relevant improvement in the absorption of fat. These results indicate

that the increased body weight in the CFTR knockout mice may be explained by a decrease in energy requirements by treating the intestinal mucosal abnormalities and/or by improving the absorption of other macro-nutrients. A drawback of the CF mouse models is that they exhibit a relatively mild fat malabsorption. It is possible that when fat malabsorption is more pronounced, for example by treating CF mice with a high fat diet or by the use of the CF ferret or CF pig with more severe fat malabsorption, the effect of antibiotic treatment on fat absorption could be more pronounced. This concept is especially interesting since we observed an accelerated fatty acid uptake and decreased fecal loss of bile salts in antibiotic-treated CF mice. These changes were not related to alterations in bile salt composition in gallbladder bile nor to changes in bacterial composition of the small intestine. The accelerated fat absorption was observed in both wild type and CF mice, suggesting a beneficial effect of antibiotics on the intestinal mucosa. This suggestion is supported by an interesting finding in premature non-CF infants with overt fat malabsorption in the late eighties.[44] Verkade et al. found that parenterally antibiotic treatment increased the absorption of fat by approximately 20% in these infants.

HEPATOBILIARY ASPECTS IN CYSTIC FIBROSIS

Various hepatobiliary manifestations are described in CF patients.[45] Approximately one-third of CF patients develop biochemical indications of liver disease (CFLD). Histologically CFLD is characterized by focal biliary cirrhosis. In a minority of cases (5-10%), focal biliary cirrhosis can progress to multilobar cirrhosis with portal hypertension and eventually end stage liver disease. Currently, ursodeoxycholic acid (UDCA) is the general clinical therapy that is applied for patients with CFLD. UDCA has shown to improve hepatobiliary symptoms and reduce serum transaminases in CF patients. At present, no effective therapy is available to cure, slow the progression, or prevent the onset of CFLD.

Ursodeoxycholic acid (UDCA)

In several cholangiopathies UDCA is beneficial by its choleretic activity and by reducing the hepatotoxicity of bile salts. While treatment with UDCA is applied for different cholangiopathies, including CFLD, the therapeutic action of UDCA in CF conditions remains unclear.,[46-47] *In-vitro* and *ex-vivo* studies indicated that the therapeutic effects depended on functional CFTR.[48] To test the choleretic activity of UDCA in CF conditions, we treated CFTR knockout and control mice with UDCA by acute intravenous infusion and by a chronic UDCA enriched diet (**Chapter 5**). We assessed bile flow and bile composition after gallbladder cannulation and the synthesis and pool size of cholate by using a micro scale stable isotope dilution technique. We found that UDCA administration stimulated bile flow up to ~500% and increased the hydrophilicity of the bile salt pool by biliary UDCA enrichment (80%) in both CFTR knockout and control mice. The high quantitative contribution of UDCA to the total bile salt pool was accompanied by a major decrease in cholate synthesis. These data indicate that the choleretic effects of

UDCA is independent from the presence of functional CFTR in mice *in vivo*, in contrast to previous *in vitro* en *ex vivo* indications.

A major portion of UDCA-stimulated bile flow in CFTR deficient conditions is most likely related to the activation of calcium activated chloride channels (CaCC). By performing bioelectric experiments in Ussing chambers we demonstrated that the calcium agonists *carbachol, ionomycin and (mucosal) ATP* all acted as major stimuli of transepithelial Cl^- and $HCO3^-$ secretion currents in gallbladders of both normal and CF mice (**Chapter 6**), indicating that CaCCs play a prominent role in secretagogue-induced fluid secretion in this tissue. The activity of CaCCs in gallbladders of humans is reduced in comparison with the activity observed in mice, but CaCCs can be upregulated in CF patients with severe liver disease.[49] Therefore, UDCA may potentially induce bile flow in CF patients through the stimulation of CaCCs rather than CFTR.

Hepatotoxicity of bile is determined by the concentration and hydrophobicity of bile salts (toxic) and by the presence of biliary phospholipids and bicarbonate (protective).[50-51] It has been suggested that the relative hydrophobic bile salt pool in CF patients contributes to the pathogenesis of CFLD.[51] Therefore, the UDCA-induced increase in the hydrophilicity of bile and the corresponding decrease of the hydrophobic bile salts in CFTR knockout mice is encouraging (**Chapter 5**). Previous studies indicate, that the phospholipid to bile salt ratio is not decreased in CF conditions. Furthermore, it has not been reported that UDCA does influence the phospholipid to bile salt ratio.[46-47] Whether UDCA also stimulates bicarbonate secretion in CFTR deficient conditions remains to be determined. It has been shown that activation of CaCC's does induce bicarbonate secretion in human CF bile-ductular cells.[52] In comparison with UDCA, norUDCA, a homologue of UDCA with one methyl group less in its side-chain, is expected to induce a more bicarbonate-rich hypercholeresis by cholehepatic shunting. The protective role of the so called "*bicarbonate umbrella*" has been investigated *in vivo* in CFTR deficient conditions. In CFTR knockout mice, norUDCA indeed induced bicarbonate secretion and stimulated bile flow two to three fold. [53] Although the effect of UDCA on bile flow was preserved in CFTR knockout mice, its effect on an injured hepatobiliary tract in CF conditions remains to be determined. The recently generated CF pig may offer a highly useful model for such studies. In addition, randomized control trials with a long-term follow-up are necessary in CF patients to evaluate the therapeutic and/or preventive effect of UDCA on CFLD.

Calcium activated chloride channels (CaCC)

A marked difference in the severity of the CF phenotype exists between mice and pigs with CF, i.e. severe in pigs but relatively mild in mice. This phenotypic difference is especially prominent in the gallbladder. CF pigs display a micro-gallbladder at birth, while CF mice have no gallbladder abnormalities.[54-57] We postulated that this difference might be due to activity of compensatory, non-CFTR chloride channels. In Ussing chambers, we measured transepithelial currents (representing anion secretion

[Cl^- and $HCO3^-$]) in gallbladders from CF pigs, CF mice and their respective wild type controls. We found that the activity of calcium activated chloride channels (CaCC) indeed corresponded with the CF gallbladder phenotype (**Chapter 6**). Induction of CaCC secretion by calcium-linked secretagogues was nearly absent in pigs, but very prominent in mice. Carbachol-induced anion secretion was upregulated under CF conditions in mice, resulting in wild type levels of total anion secretion (the sum of CFTR and CaCC induced secretions). However, it should be noted that the carbachol-induced anion secretion, in contrast to CFTR activity, is a relatively short and transient event. Moreover, the relative contribution of Cl^- and $HCO3^-$ ions to the anion secretion was not addressed in our study. Therefore, we recommend for future studies to investigate (1) whether the CaCC-mediated anion secretion, in view of its transient nature, still has a sufficient impact on osmotically driven fluid secretion; (2) what proportion of the anion current is carried by bicarbonate. Recent reports highlight the importance of bicarbonate secretion in the formation of normal mucus[58-60] and thus, this is of special interest in CF conditions where impaired bicarbonate secretion is present.

Previous study suggested that the CaCC TMEM16A is the most important CaCC in the apical membrane of epithelial tissues.[61] Immunostaining of TMEM16A, showed a pronounced staining intensity in the mouse gallbladder epithelium and a relatively weak staining intensity in the pig gallbladder, matching the functional data on anion secretion (**Chapter 6**). The TMEM16A mRNA expression levels did not match the functional data and unfortunately, sufficient TMEM16A antibodies for western blotting, to quantitate protein expression levels more accurately, were lacking. Since TMEM16A knockout mice die shortly after birth,[62] a gallbladder TMEM16A knockout, or crossings of this KO with CFTR-/- mice, should be developed to investigate whether the loss of TMEM16A anion channels alone (or in combination with CFTR deficiency) is sufficient to develop a micro-gallbladder and other hepatobiliary abnormalities present in CF patients. Another option for future studies includes the use of specific TMEM16A inhibitors in CF and wild type mice and the use of TMEM16A activators in CF pigs to test whether the hepatobiliary phenotype will deteriorate or improve respectively.

The activation of CaCCs may potentially migitate the hepatobiliary phenotype in CF patients, by compensating for the loss in CFTR function and thereby providing an alternative for targeting defective CFTR. In comparison with CFTR correctors or potentiators, an important theoretical advantage of the development of CaCC-activators is that they are expected to be applicable universally in all CF mutation classes and thus, its treatment will not be restricted to subsets of CF patients. CaCC activation also has disadvantages in comparison to CFTR correctors or potentiators. First, CFTR function comprises much more than "only" fluid transport and thus, the other regulatory functions of CFTR will not be accounted for when stimulating CaCCs. Second, as mentioned earlier, channel activity of CaCC is characterized by a transient channel opening, while CFTR is characterized by a a more stable opening of the channel (hours rather than minutes). Third, CaCCs are not equally expressed in all epithelial tissues that are affected in CF

patients. For example, CaCCs are not expressed in the intestine (what could contribute to the severe intestinal phenotype of most CF mouse models).[63] Finally, TMEM16A and other CaCCs are expressed too in non-epithelial tissues, e.g. smooth muscle cells and Cajjar cells[61], implying that pharmacological CaCC activators may exert unwanted side-effects that may restrict their use as oral or IV medication.

SUMMARY AND OVERALL CONCLUSION

The aim of our thesis was to address various aspects of the gastro-intestinal tract in CF disease by evaluating current treatment and investigating potential future treatment options. Optimizing nutritional status is a classic cornerstone in treating CF patients. The definition of an '*optimal nutritional status*' in school-aged CF patients with preserved pulmonary function may, to our understanding, be less stringent than currently applied. Our data indicate that the current strive for z-scores for BMI above zero to maintain a good pulmonary function (FEV1 >80%) is not necessary, as 50% of our patient population have a preserved pulmonary function despite z-scores for BMI below zero. In addition, we recommend to re-evaluate the strive for high dietary energy intakes in relatively healthy CF patients. Our retrospective cross-sectional and longitudinal analysis revealed a very weak relation between energy intake and parameters for nutritional status and growth in patients with preserved pulmonary function. Prospective clinical trials are indicated in this patient group to investigate the exact quantitative contributory effect of high energy intakes on nutritional status and growth. Clarity in this patient group is important, in order to determine whether the (beneficial) effects on nutritional status outweigh the potentially negative psychological burden for CF patients to comply to the nutritional advice. Another therapeutic target to improve nutritional status includes the correction of (persistent) fat malabsorption in CF patients. Acid suppressive drugs may improve fat absorption in individual CF patients. Broad-spectrum antibiotic therapy improves the nutritional status of CFTR knockout mice with small intestinal bacterial overgrowth, but not via improving the absorption of fat. Antibiotic treatment did accelerate the absorption of fat, which raises the question whether changes in fat absorption may be observed in CF patients or CF animal models with more overt fat malabsorption (e.g. the CF ferret or CF pig) than the CF mice. The contribution of essential fatty acid deficiency and prolonged transit time on fat malabsorption in CF patients remains to be determined, although we have not found indications that these factors profoundly interfere with fat absorption in CF patients. We demonstrated that the beneficial effects of UDCA on bile flow and bile composition are maintained under CF deficient conditions, indicating that these effects are CFTR-independent. These results encourage the need to investigate the therapeutic and/or preventive effect of UDCA in CF-related liver disease in prospective randomized clinical trials. Finally, the absent gallbladder phenotype in CF mice, associated with a pronounced capacity of calcium induced anion secretion across non-CFTR anion channels, strengthens the basis for exploiting pharmacological activation of calcium activated chloride channels as a therapeutic strategy to prevent or mitigate the CF phenotype.

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